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Hepatic uptake of cholecystokinin octapeptide by organic anion-transporting polypeptides OATP4 and OATP8 of rat and human liver

Ismair, M G ; Stieger, B ; Cattori, V ; Hagenbuch, B ; Fried, M ; Meier, P J ; Kullak-Ublick, G A

Abstract: **BACKGROUND** AIMS: Cholecystokinin (CCK) is a major gastrointestinal peptide hormone that is released postprandially from the small intestine and exerts marked effects on gallbladder and gastrointestinal motility. The smaller isoforms CCK-8 and CCK-4 are rapidly taken up into hepatocytes, metabolized, and excreted into bile. Our aim was to identify and characterize the hepatocellular CCK-8 uptake system. **METHODS:** CCK-8 uptake was measured in *Xenopus laevis* oocytes expressing the organic anion-transporting polypeptides of rat liver (Oatp1, Oatp2, Oatp3, or Oatp4) and of human liver (OATP-A, OATP-B, OATP-C, or OATP8) and in primary cultured rat hepatocytes. **RESULTS:** Rat Oatp4 and human OATP8 efficiently mediated CCK-8 uptake in oocytes, with Michaelis constant (K_m) values of 14.9 ± 2.9 micromol/L and 11.1 ± 2.9 micromol/L, respectively. CCK-8 uptake by hepatocytes was also saturable, with a K_m of 6.7 ± 2.1 micromol/L. The K_m value in rat hepatocytes is consistent with Oatp4-mediated transport. **CONCLUSIONS:** CCK-8 is selectively transported by rat Oatp4 and human OATP8, both of which are exclusively expressed at the basolateral membrane of hepatocytes. These 2 transporters are the first and probably the predominant hepatic uptake systems for CCK-8 and may be critical for the rapid clearance of this hormone from the circulation.

DOI: <https://doi.org/10.1053/gast.2001.28704>

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ZORA URL: <https://doi.org/10.5167/uzh-3000>

Journal Article

Accepted Version

Originally published at:

Ismair, M G ; Stieger, B ; Cattori, V ; Hagenbuch, B ; Fried, M ; Meier, P J ; Kullak-Ublick, G A (2001). Hepatic uptake of cholecystokinin octapeptide by organic anion-transporting polypeptides OATP4 and OATP8 of rat and human liver. *Gastroenterology*, 121(5):1185-1190.

DOI: <https://doi.org/10.1053/gast.2001.28704>

MS: 00-1716-00

Hepatic Uptake of Cholecystokinin Octapeptide (CCK-8) by Organic Anion Transporting Polypeptides Oatp4 (Slc21a6) and OATP8 (SLC21A8) of Rat and Human Liver

Short title: CCK-8 uptake by hepatic OATPs

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Grant support: Supported by grant 32-59155.99 from the Swiss National Science Foundation, Bern/Switzerland.

The authors thank Monika Gersbach-Fey for expert technical assistance.

Abbreviations: CCK, cholecystokinin; BSP, bromosulphophthalein; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; DPDPE, [D-penicillamine^{2,5}]enkephalin; LST-1, liver specific transporter-1; LTC₄, leukotriene C₄; Oatp/OATP, rat/human organic anion transporting polypeptide; PGE₂, prostaglandin E₂; SLC, solute carrier

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Abstract

Background & Aims: Cholecystokinin (CCK) is a major gastrointestinal peptide hormone which is released postprandially from the small intestine and which exerts marked effects on gallbladder and gastrointestinal motility. The smaller isoforms CCK-8 and CCK-4 are rapidly taken up into hepatocytes, metabolized and excreted into bile. Our aim was to identify and characterize the hepatocellular CCK-8 uptake system. **Methods:** CCK-8 uptake was measured in *X. laevis* oocytes expressing the organic anion transporting polypeptides of rat (Oatp1, Oatp2, Oatp3 or Oatp4) and human liver (OATP-A, OATP-B, OATP-C or OATP8), and in primary cultured rat hepatocytes. **Results:** Rat Oatp4 and human OATP8 efficiently mediated CCK-8 uptake in oocytes, with Km values of $14.9 \pm 2.9 \mu\text{M}$ and $11.1 \pm 2.9 \mu\text{M}$, respectively. CCK-8 uptake by hepatocytes was also saturable, with a Km of $6.7 \pm 2.1 \mu\text{M}$. The Km value in rat hepatocytes is consistent with Oatp4 mediated transport. **Conclusion:** CCK-8 is selectively transported by rat Oatp4 and human OATP8, both of which are exclusively expressed at the basolateral membrane of hepatocytes. These two transporters are the first and probably the predominant hepatic uptake systems for CCK-8 and may be critical for the rapid clearance of this hormone from the circulation.

Introduction

Cholecystokinin (CCK) is a gastrointestinal peptide hormone with a wide range of biological activities. CCK is released postprandially from the intestinal wall and stimulates gallbladder contraction, the release of pancreatic enzymes and intestinal motility ¹. Gastric emptying is delayed by CCK ², whereas CCK-A receptor blockade accelerates gastric emptying ³. Among the various CCK isoforms the sulfated C-terminal octapeptide (CCK-8) has repeatedly been reported to be biologically more active than CCK-39 or CCK-33 ⁴⁻⁹, but other studies indicate that CCK-8 is not more potent than its larger analogues ¹⁰⁻¹². Although CCK-8 shows no metabolic effect on hepatocytes it is efficiently taken up from portal blood into liver cells ^{13,14}, where it is rapidly metabolized and finally excreted into bile ¹⁵.

To date little is known about the molecular mechanism of hepatocellular CCK-8 uptake. The existence of an efficient, highly specific and saturable transport system has been postulated and it has been shown that hepatic CCK-8 uptake is sodium-independent and can be inhibited by DIDS, the organic anion bromosulphophthalein (BSP) and the bile salt taurocholate ^{14,16}. These transport characteristics are typical for the organic anion transporting polypeptides (Oatps in rodents and OATPs in man), a family of plasma membrane carriers which are classified as *SLC21A* within the gene superfamily of solute carriers (Human Gene Nomenclature Committee Database). Oatps/OATPs are multispecific transporters that mediate the hepatic uptake of numerous amphipathic albumin bound compounds including bile salts, organic anions (e.g. BSP), conjugated steroids, eicosanoids, cardiac glycosides (e.g. ouabain and digoxin) and thyroid hormones. Interestingly, the Oatps/OATPs have also been shown to transport certain oligopeptides and peptidomimetic drugs,

including the thrombin inhibitor CRC 220^{17,18}, the opioid peptides [D-penicillamine^{2,5}]enkephalin (DPDPE) and deltorphin II, and the cyclic endothelin antagonist BQ-123¹⁹⁻²¹.

Recently, a new liver specific Oatp/OATP subfamily has been identified that is expressed exclusively at the basolateral membrane of hepatocytes. The human OATP-C (*SLC21A6*) is also termed “liver-specific transporter 1” (LST-1) or OATP2²²⁻²⁴ and is 64% identical with the rat homologue Oatp4 (*Slc21a6*)²⁵. Whereas Oatp4 is the only member of this subfamily identified in rat liver, human hepatocytes – in addition to OATP-C – also express OATP8 (*SLC21A8*), which is 80% and 66% identical with human OATP-C and rat Oatp4, respectively²⁶. We have recently shown that OATP8 appears to be particularly efficient in the transport of certain peptides²¹.

In this study we show that hepatocellular CCK-8 uptake is indeed an Oatp/OATP mediated process. However, CCK-8 is selectively transported by Oatp4 in rat liver and OATP8 in human liver, indicating that these two transporters may be critical for CCK-8 clearance from the portal and systemic circulation.

Materials and Methods

Materials. Radiolabeled [^3H]CCK-8 (69 - 74 Ci/mmol) was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). Collagenase type 2 CLS was from Worthington Biochemical Corp. (Freehold, NJ). All other chemicals were of the highest degree of purity available and were readily available from commercial sources.

Uptake studies in *Xenopus laevis* oocytes. Capped Oatp/OATP cRNA was synthesized *in vitro* using the mMESSAGE mMACHINETM T7 kit (Ambion, Austin, TX) as described ²¹. *X. laevis* oocytes were prepared as described ²⁷ and cultured overnight at 18°C. Healthy oocytes were microinjected either with 5 ng of the respective cRNA in a volume of 50 nl or with 50 nl of water and were subsequently cultured for 3 days in modified Barth's solution at pH 7.6 ²⁸. All uptake studies were performed in a sodium-free medium containing 100 mM choline chloride, 2 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , and 10 mM HEPES adjusted to pH 7.5 with Tris. Oocytes were prewashed in uptake medium and then incubated at 25°C in 100 μl of uptake medium containing radiolabeled CCK-8 at the indicated concentrations. Water injected oocytes were used as controls for nonspecific uptake and binding of the substrate. Uptake was stopped by the addition of 6 ml ice-cold uptake medium and oocytes were washed in 2 x 6 ml of ice-cold uptake medium. Individual oocytes were lysed in 0.25 ml of 10% (w/v) SDS and 4 ml scintillation fluid (Ultima Gold; Canberra Packard, Zurich, Switzerland), and the oocyte associated radioactivity was determined in a Tri-Carb 2200 CA liquid scintillation analyzer (Canberra Packard).

Isolation of rat hepatocytes. Male Sprague-Dawley rats (200 - 250 g body weight) were anesthetized with sodium pentobarbital (Nembutal, Abbot, North Chicago, IL, 50mg/kg body weight, intraperitoneally). Hepatocytes were isolated according to a slightly modified 2-step collagenase perfusion method as previously described ²⁹. Cell viability was assessed by trypan blue exclusion and was more than 80 %. 2.4×10^6 cells were seeded into 60 mm petridishes in Williams medium E supplemented with 2 mmol/l glutamine 100 U/ml penicillin, 100 µg/ml streptomycin, 250 nU/ml insulin, 1 µmol/l dexamethasone, and 10 % (v/v) fetal calf serum. Hepatocytes were allowed to attach for 3-4 hours at 37°C in a humidified atmosphere containing 5 % CO₂.

Uptake studies in hepatocytes. Immediately prior to uptake, the cell monolayer was washed 3 times with 3 ml of prewarmed (37°C) or ice-cold (4°C) uptake buffer consisting of 116 mmol/l NaCl, 5.3 mmol/l KCl, 1.1 mmol/l KH₂PO₄, 0.8 mmol/l MgSO₄, 1.8 mmol/l CaCl₂, 11 mmol/l D-glucose, and 20 mmol/l HEPES, pH 7.4. Uptake was started by the addition of 2 ml of uptake buffer (37° or 4°C) containing [³H]CCK-8 at the indicated concentrations. Uptakes were stopped by aspiration of the uptake buffer and four rapid washes with 3 ml of ice-cold substrate-free uptake buffer. Cells were lysed in 2 ml of 1 % (v/v) Triton X-100. 1 ml of the lysate was mixed with 5 ml of scintillation fluid (Ultima Gold; Canberra Packard, Zurich, Switzerland), and cell associated radioactivity was determined in a Tri-Carb 2200 CA liquid scintillation analyzer (Canberra Packard). Protein content of the lysates was measured with the bicinchoninic acid method (Pierce, Pockford, IL) using bovine serum albumin in 0.5 % (v/v) Triton X-100 as standard.

Statistical analysis. Uptake results are given as the mean \pm one standard deviation (SD). Differences in uptake between cRNA and water injected oocytes were tested for statistical significance using the unpaired Student *t* test. Statistical significance was assumed at $p < 0.05$. Kinetic parameters of uptake were calculated according to the Michaelis-Menten equation $v = V_{\max} \cdot [S] / (K_m + [S])$.

Results

We initially tested transport of [^3H]CCK-8 by the known Oatps of rat liver. Three days after injection of cRNA into *X. laevis* oocytes, the liver specific Oatp4 (*Slc21a6*) efficiently mediated CCK-8 transport (25 fold stimulation of uptake as compared to water injected oocytes), whereas oocytes expressing either Oatp1 (*Slc21a1*), Oatp2 (*Slc21a5*) or Oatp3 (*Slc21a7*) did not show any measurable uptake (Fig. 1A). We next tested the four OATPs expressed in human liver for their ability to transport CCK-8. Interestingly, only OATP8 (*SLC21A8*) efficiently transported CCK-8 (8 fold stimulation of uptake compared to water injected oocytes), whereas OATP-A (*SLC21A3*), OATP-B (*SLC21A9*) and OATP-C (*SLC21A6*) did not (Fig. 1B). CCK-8 was thus shown to be transported selectively by Oatp4 of rat liver and OATP8 of human liver.

We next analyzed the kinetic characteristics of Oatp4 and OATP8 mediated CCK-8 transport. To determine the initial linear phase of CCK-8 uptake, time course experiments were performed using one low (0.5 $\mu\text{mol/l}$) and one high (100 $\mu\text{mol/l}$) concentration. At both substrate concentrations, uptake in Oatp4 and OATP8 expressing oocytes increased linearly over at least 40 minutes, whereas nonspecific uptake and/or binding to water injected control oocytes did not increase with time (Figs. 2A and 2B). We therefore performed kinetic studies over a period of 20 minutes using CCK-8 concentrations in the range of 0.5 to 100 μM . Oatp4 mediated initial uptake rates were saturable with an apparent K_m value of $14.9 \pm 1.5 \mu\text{M}$ and a V_{max} of $88.7 \pm 2.6 \text{ fmol/oocyte}\cdot\text{min}$ (Fig. 3A). OATP8-mediated CCK-8 uptake was also saturable, with an apparent K_m value of $11.1 \pm 2.9 \mu\text{M}$ and a V_{max} of $55.1 \pm$

4.0 fmol/oocyte·min (Fig. 3B). These results indicated highly comparable kinetic features for CCK-8 transport by the rat and human transporters.

To correlate the kinetic features of Oatp4/OATP8 mediated CCK-8 uptake in oocytes with uptake in liver, CCK-8 uptake by primary cultured rat hepatocytes was measured. The specific protein-mediated portion of uptake was determined by subtracting uptake values at 4°C from those measured at 37°C. Initial uptake at concentrations of up to 30 μ M was linear over at least 1 minute (data not shown). Kinetic measurements using concentrations between 1-30 μ M showed saturability of CCK-8 uptake, with an apparent K_m value of 6.7 ± 2.1 μ M and a V_{max} of 27.7 ± 2.7 fmol/mg x 20 seconds (Fig. 4). These data indicate that the affinity of Oatp4 for CCK-8 is identical with that of the CCK-8 uptake system in rat hepatocytes.

Discussion

Over the past two decades increasing evidence has suggested carrier-mediated hepatic uptake of the gastrointestinal peptide hormone CCK-8, including (i) a concentration gradient for this hormone between the portal and the systemic venous circulation ³⁰, (ii) a reduced biological activity of CCK-8 after intraportal administration as compared to systemic administration ³¹, (iii) elevated CCK-8 plasma levels in patients with liver cirrhosis ³², (iv) rapid appearance of radiolabeled CCK-8 in the liver after intravenous injection, as shown by whole body autoradiography ³³, and (v) uptake studies with radiolabeled CCK peptides in the isolated perfused rat liver and in primary cultured rat hepatocytes ¹⁴⁻¹⁶.

In the present study, we show that CCK-8 is efficiently transported by Oatp4 of rat liver and OATP8 of human liver, whereas all other hepatic Oatps/OATPs did not transport this peptide. These findings are of particular interest for two reasons. First, they confirm previous functional studies, which had identified an Oatp-like transport system for CCK-8 in hepatocytes ¹⁴⁻¹⁶. Second, they identify CCK-8 as a specific physiological substrate of rat Oatp4 and human OATP8. It is noteworthy that OATP8 but not OATP-C transports CCK-8, considering that these two carriers share an 80 % amino acid identity and exhibit closely overlapping substrate specificities including certain peptides ²¹. The results further confirm the notion that OATP8 is an uptake system for oligopeptides, whereas OATP-C is the more efficient transporter for organic anions including bilirubin ³⁴.

The results of this study suggest that Oatp4 is the predominant CCK-8 transport system of rat hepatocytes. This conclusion is based on the following observations. First, Oatp4 transports CCK-8 in the absence of sodium and hepatocellular uptake of CCK-8 was shown to be sodium-independent ¹⁴. Second, kinetic studies of Oatp4 mediated CCK-8 transport yielded an apparent K_m value of $14.9 \pm 1.5 \mu M$. This would seem to be a low affinity if one considers that plasma CCK-8 concentrations are in the picomolar range. However, the data are in perfect agreement with the K_m value of $6.7 \pm 2.1 \mu M$ that was determined for CCK-8 uptake in primary cultured rat hepatocytes. The hepatocellular affinity for small CCK peptides such as CCK-4 also appears to be in the same range, since CCK-4 has been shown to inhibit hepatocellular CCK-8 uptake with an apparent K_i value of $63 \mu M$ ¹⁴. Third, CCK-8 uptake by rat hepatocytes has been shown to be competitively inhibited by the organic anion BSP and the bile salt taurocholate with apparent K_i values of $6.2 \mu M$ and $17 \mu M$, respectively ¹⁴. This is in agreement with our recent findings that Oatp4 mediates uptake of BSP and taurocholate with apparent K_m values of $1.1 \mu M$ and $27 \mu M$, respectively ²⁵. We therefore conclude that Oatp4 is the low affinity / high capacity CCK-8 uptake system of rat hepatocytes postulated by Gores and coworkers ^{14,16}.

In summary, we have shown that the hepatocellular uptake system for CCK-8 is Oatp4 in rats and most likely OATP8 in man. The selective transport of CCK-8 by rat Oatp4 and human OATP8 but not by other Oatps/OATPs suggests that intact function of Oatp4 and OATP8 may be critical for CCK-8 clearance from the circulation following its postprandial release from the intestine.

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Figure legends

Fig. 1: Comparison of CCK-8 uptake by *X. laevis* oocytes expressing either (A) rat Oatps or (B) human OATPs. Oocytes were injected with 5 ng cRNA coding for the respective Oatp/OATP or with 50 nl water. After 3 days in culture, initial uptake of 0.2 $\mu\text{mol/l}$ [^3H]CCK-8 was measured over 30 minutes as described in “Materials and Methods”. Bars represent the mean \pm SD of 8-12 separate oocyte measurements. *statistically significant uptake by Oatp/OATP cRNA injected oocytes compared to water injected control oocytes.

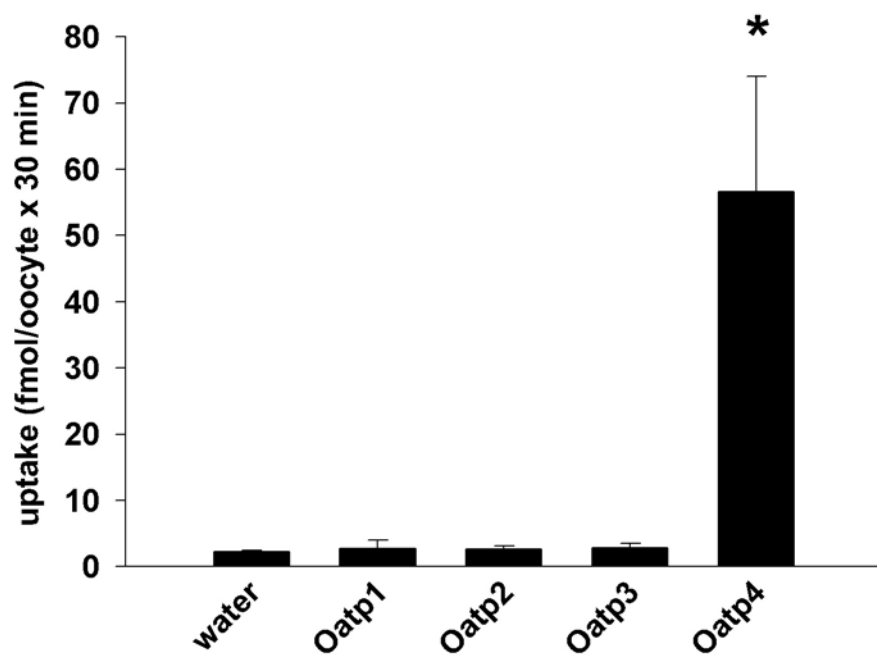
Fig. 2: Time course of CCK-8 uptake by oocytes injected with Oatp4 cRNA (o), OATP8 cRNA (●) or water (▲). Uptake was measured at (A) 0.5 $\mu\text{mol/l}$ and (B) 100 $\mu\text{mol/l}$. Individual data points show mean \pm SD of 8-12 separate oocyte measurements.

Fig. 3: Kinetics of CCK-8 uptake in (A) Oatp4 and (B) OATP8 cRNA injected *X. laevis* oocytes. Oocytes were injected with 5 ng cRNA or with 50 nl water. After 3 days in culture, uptake of [^3H]CCK-8 was measured over 20 minutes at the indicated concentrations. Uptake values represent mean \pm SD of 8-12 separate oocyte measurements. Oatp/OATP specific uptake (-----) was calculated by subtracting nonspecific uptake in water injected oocytes (o—o) from uptake in cRNA injected oocytes (●—●). Curves were fitted by non-linear regression analysis.

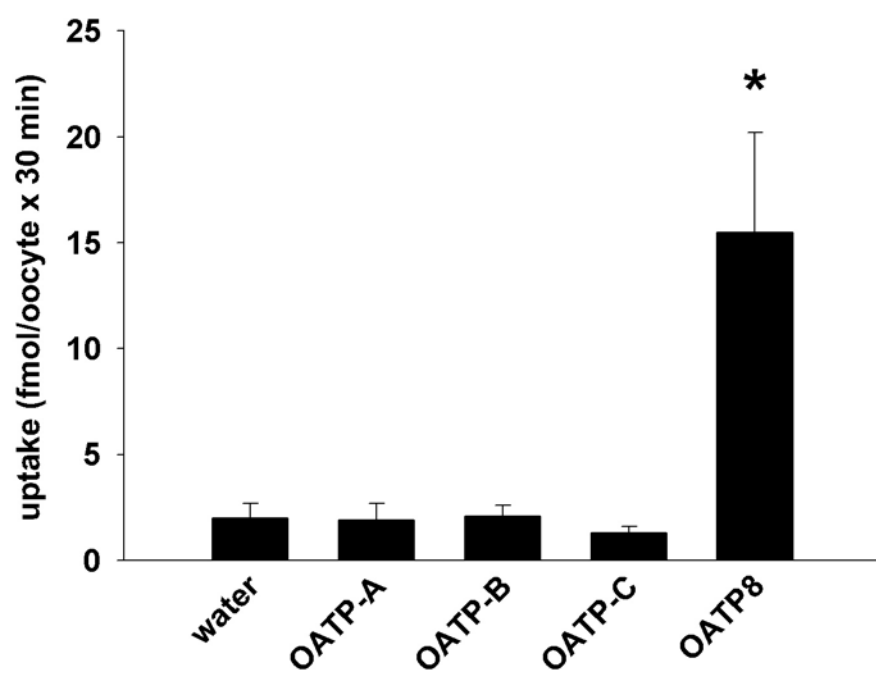
Fig. 4: Kinetics of CCK-8 uptake in primary cultured rat hepatocytes. Hepatocytes were cultured for 3 hours and uptake of [^3H]CCK-8 was measured over 20 seconds

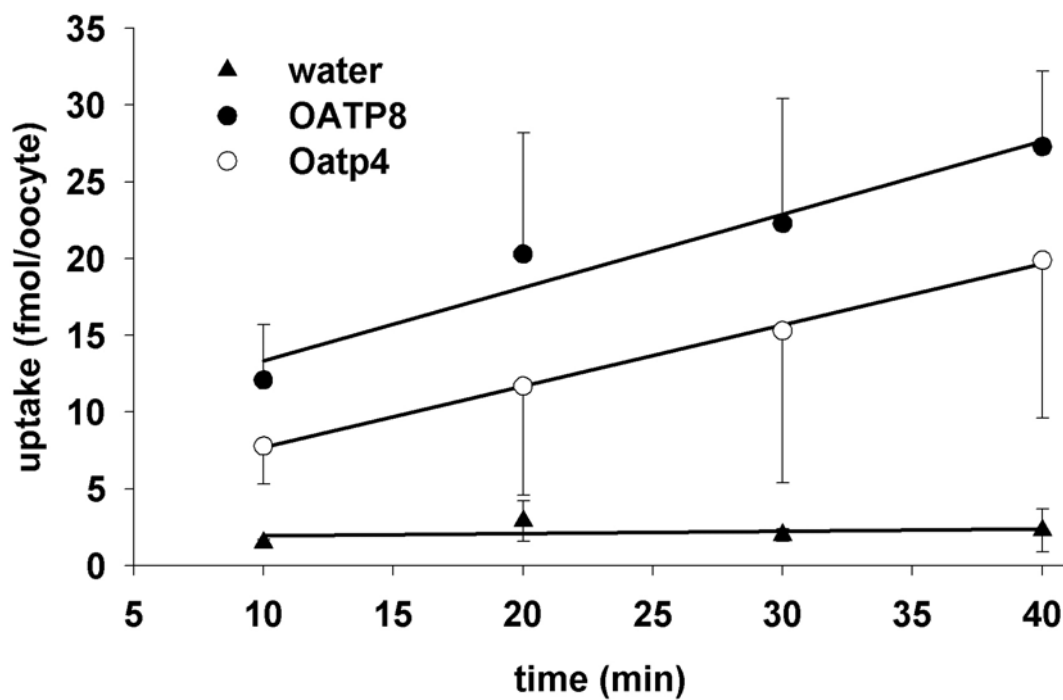
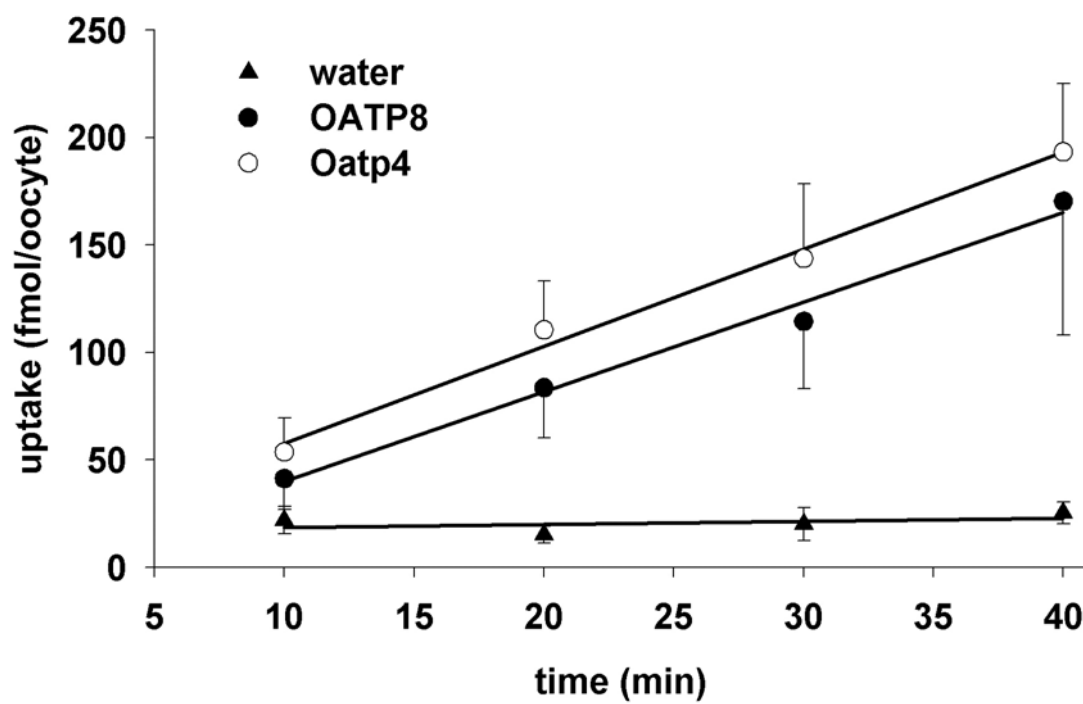
at the CCK-8 concentrations indicated. Uptake values represent net protein-mediated uptake calculated by subtracting nonspecific uptake at 4°C from uptake at 37°C. Data are shown as the mean value of triplicate uptake experiments and the curve was fitted by non-linear regression analysis.

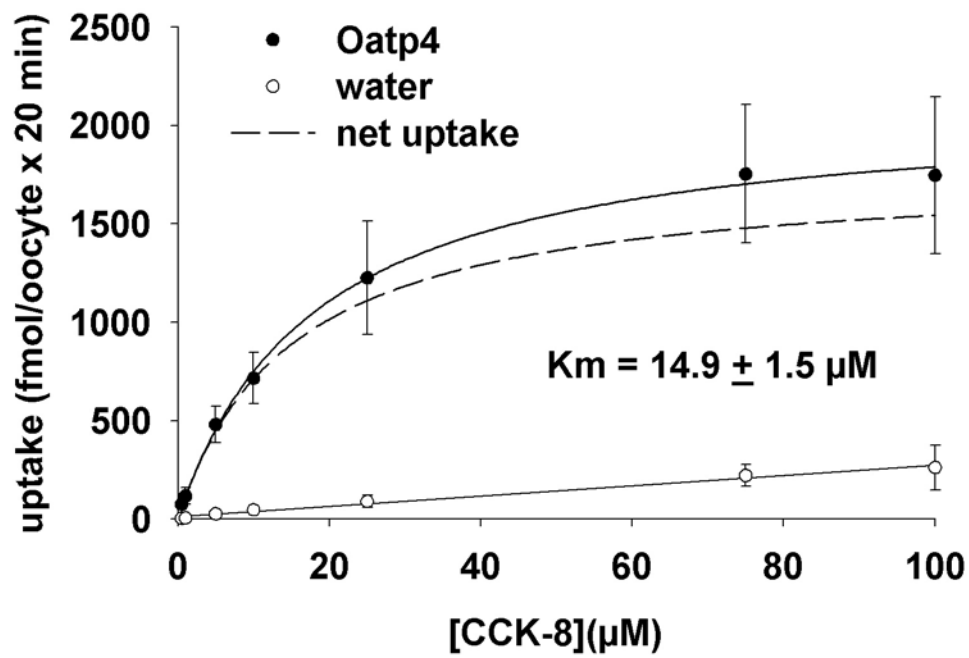
A



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A**B**

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